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LABORATORY INVESTIGATION

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Immunohistochemical Localization of Keratin in Normal Human Tissues

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Immunohistochemical identification of intracellular keratin was achieved using an indirect antibody technique on paraffin-embedded human tissue. A study of numerous tissues confirms that keratins are abundant in all layers of squamous epithelia, in the ducts of epithelial-derived glands, and in the epithelia of the respiratory and urinary tracts. Using an immunoperoxidase technique which offers increased histologic resolution, we have shown that the basal or reserve cells of the tracheal, bronchial, prostatic, and cervical gland epithelia are the predominant keratin-containing cells in these tissues. The normal differentiation of basal cells into nondividing, superficial columnar cells is accompanied by the loss of cytoplasmic keratin proteins. Foci of epithelial squamous metaplasia stain intensely with antikeratin antibodies and presumably represent an exaggerated proliferation of the keratin-containing basal cells. Alveolar respiratory epithelium, acinar cells of various glands, and many mesodermal tissues (muscle, hematopoietic, and lymphoid tissue, nerve, and connective tissue) were devoid of keratin proteins. The ability to identify keratin proteins within fixed, embedded tissue (including those known to lack tonofilament bundles) may prove useful in the study of tissue histogenesis and carcinogenesis, and in the pathologic assessment of poorly differentiated malignant neoplasms and tumors of controversial cellular origin.

Additional key words: Keratin proteins, Epithelial differentiation, Squamous metaplasia, Immunoperoxidase staining.

During differentiation, epidermal cells synthesize intracytoplasmic filamentous proteins which aggregate into characteristic tonofilament bundles. The individual filaments have an approximate diameter of 8 nm. and are often noted in intimate association with desmosomes (23), presumably reflecting a role in the structural integrity of skin. Purified epidermal keratin proteins (which range from 40,000 to 65,000 molecular weight), can participate in the *in vitro* assembly of 8-nm. filaments (25), and antibodies prepared against a specific 58,000-molecular weight epidermal protein have been shown to localize ultrastructurally to the 8-nm. tonofilaments of epidermis (5, 12).

Only recently has it been recognized that keratin filaments are not restricted to epidermis (8, 9, 27, 29). Franke, Schmid, Osborn, and Weber (9) demonstrated that *in vitro* cultures of epithelia such as human kidney and bovine mammary epithelium stained with antikeratin antibodies with a typical cytoplasmic, filamentous pattern. When frozen sections of rodent tissue were stained with the same antibody, fluorescence was detected in the myoepithelium of the rat mammary gland and in the biliary duct cells of the mouse (8). Sun and Green (27) and Sun, Shih, and Green (29) studied keratin distribution in various rabbit and some human tissues by immunofluorescence microscopy and clearly defined many types of nonsquamous epithelium which contained keratin (*e.g.*, trachea, cervix, intestine, and bladder) as well as those tissues which were free of keratin (*e.g.*, muscle, nerve, and cartilage).

The present study surveys human tissues for keratin proteins with the use of an immunoperoxidase technique. Our results confirm the presence of keratin in a variety of nonsquamous cells, but reveal an intraepithelial localization of keratin in human lung and in glands which is somewhat different from that described in the rabbit. In addition, the method should prove to be useful in the pathologic analysis of poorly differentiated human tumors.

MATERIALS AND METHODS

ANTIGEN PREPARATION

Keratin proteins were isolated according to the method of Sun and Green (28). Stratum corneum (600 mg.) obtained from the sole of the human foot was homogenized in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, until the tissue was thoroughly dispersed. The suspension was centrifuged at 12,000 r.p.m. (17,300 × *g*) for 20 minutes in a Sorvall RC2-B centrifuge. The pellet was extracted twice with 20 mM Tris-HCl, pH 7.4, and three times with 20 mM Tris-HCl, pH 7.4, con-

taining 8 M urea to remove water-soluble proteins, leaving the water-insoluble keratin in the pellet. The insoluble pellet was then extracted with 20 mM Tris-HCl, pH 7.4, containing 8 M urea and 10 mM dithiothreitol (to solubilize the disulfide cross-linked protein), sonicated for 15 seconds at a setting of 5 on a Bronson sonifier and centrifuged. The supernatant fluid was used as antigen.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Purified keratin protein (30 μg.) was applied to a 10 per cent polyacrylamide gel with a stacker gel of 5 per cent polyacrylamide. Both gels contained 0.1 per cent sodium dodecyl sulfate, essentially as described by Laemmli (15). Electrophoresis was performed at 30 ma. until the tracking dye reached the bottom of the gel (4 hours). After staining with Coomassie blue dye, keratin proteins varying from 41,000 to 65,000 daltons could be identified (Fig. 1). Similar patterns have been obtained by Sun and Green (28).

ANTIBODY PREPARATION

Keratin proteins purified from human stratum corneum as described above were emulsified with Freud's complete adjuvant and injected intradermally into New Zealand White rabbits, using methods described previously (28). One milligram of keratin protein was used for the primary injection. One month later, a second injection containing 0.5 mg. of protein was given. Serum was collected 7 to 10 days after the second injection. Double diffusion studies in 1 per cent agarose gels containing 0.1 per cent sodium dodecyl sulfate and 0.5 per cent Triton X-100 (30) revealed a single, strong precipitin band between the antiserum and keratin proteins extracted from stratum corneum. Preabsorption of the antiserum with keratin proteins eliminated this band; 0.6 ml. of antiserum was preabsorbed by overnight incubation at 37° C. with keratin extracted from 0.2 gm. of human stratum corneum. There was no immunologic cross-reactivity of immune or preimmune serum with proteins of cultured 3T3 fibroblasts or fibroblasts derived from human skin. Prior to use in immunoperoxidase studies, the preimmune and immune sera were adsorbed with mouse liver powder to minimize nonspecific staining.

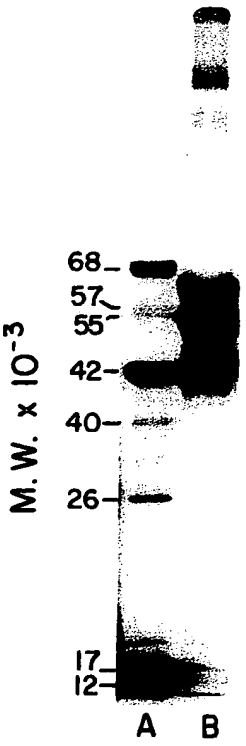

FIG. 1. Sodium dodecyl sulfate gel electrophoresis pattern of keratin proteins selectively extracted from human stratum corneum. Molecular weight standards on the left side of the gel are: bovine serum albumin (68,000), tubulin (57,000 and 55,000), actin (42,000), aldolase (40,000), chymotrypsinogen (26,000), myoglobin (17,000), and cytochrome *c* (12,000).

IMMUNOPEROXIDASE STAINING

Paraffin-embedded tissues were retrieved from the surgical pathology files of the Peter Bent Brigham Hospital, and immunoperoxidase staining of these tissues was performed according to a modification (21) of published methods (16, 18, 26). Unless otherwise specified, all tissues were fixed in 10 per cent buffered formalin. Sections (5 μm. thick) were transferred to glass slides and warmed to 45° C. for 1 hour to ensure adherence. The sections were deparaffinized in xylene, placed in absolute alcohol, then sequentially incubated for 30 minutes at room temperature with each of the following reagents: (1) methanolic hydrogen peroxide (1 volume of 3 per cent aqueous hydrogen peroxide to 5 volumes of methanol); (2) rabbit antihuman keratin antiserum (1:20 to 1:100 dilution); (3) swine antirabbit serum IgG (1:20 dilution); (4) horseradish peroxidase-rabbit antihorseradish peroxidase-soluble complexes (1:100 dilution). All dilutions were performed with 0.1 M Tris-buffer, pH 7.6. To decrease nonspecific background staining, sections were incubated with either 5 per cent egg albumin or normal swine serum (1:10 dilution) for 30 minutes following methanolic peroxide treatment. After each incubation, slides were washed with Tris-saline (1 volume Tris buffer, 0.1 M, pH 7.6, to 9 volumes normal saline), then placed in Tris buffer for 15 minutes. Antibody localization was determined by detection of peroxidase activity, effected by a 5-minute incubation of the slides with a freshly prepared solution containing 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Company, St. Louis, Missouri, 6 mg. per 10 ml. of Tris buffer) and 0.1 ml. of 3 per cent hydrogen peroxide. This method yields a brown reaction product. Slides were then washed with water, counterstained with hematoxylin, dehydrated, and mounted in Permount.

Horseradish peroxidase-rabbit antihorseradish peroxidase-soluble complexes and swine antirabbit serum IgG were purchased from Dakopatts A/S of Copenhagen, Denmark (United States agent, Accurate Chemical and Scientific Company, Hicksville, New York).

RESULTS

In Table 1 is defined the distribution of keratin proteins in a wide variety of adult human tissues. For all tissue with detectable intracellular keratin proteins, the speci-

TABLE 1. DISTRIBUTION OF INTRACYTOPLASMIC KERATIN PROTEINS IN NORMAL HUMAN TISSUES AS DETERMINED BY IMMUNOPEROXIDASE STUDIES

Tissues	Result	Tissues	Result
Skin		Pancreas	
Epidermis	+	Ducts	+
Hair follicle	+	Acini	–
Sebaceous gland		Islets	–
Sebaceous cell	(+)[a]	Breast	+[b]
Basal cell	+	Urinary bladder and ureter	
Sweat gland		Transitional epithelium	+
Ductal cells	+	Smooth muscle	–
Dermal collagen	–	Kidney	
Lung		Glomeruli	–
Trachea and bronchus		Tubules, collecting	+
Columnar epithelium	–	Tubules, proximal and distal	–
Basal cells	+	Liver	
Intermediate cells	+	Hepatocytes	–
Submucosal glands		Bile ducts	(+)[a]
Ducts	+	Hepatic arteries, veins	–
Acini	–	Testis	
Alveoli	–	Tubules	–
Cervix		Interstitium	–
Exocervix		Adrenal	–
Squamous epithelium	+	Ovary	–
Endocervix		Stomach	–[c]
Columnar epithelium	–	Intestine	
Basal cells	+	Ileum	–[c]
Vagina		Colon	–[c]
Squamous epithelium	+	Nerve	–
Prostate		Muscle	
Columnar cells	–	Smooth	–
Basal cells	+	Skeletal	–
Oral cavity		Connective tissue	–
Tongue		Lymph node	–
Squamous mucosa	+	Spleen	–
Buccal mucosa	+	Hematopoietic (iliac crest)	
Parotid gland		Erythroid, myeloid, and megakaryocytic lines	–
Ducts	+	Bone trabeculae	–
Acini	–		
Submaxillary gland			
Ducts	+		
Acini	–		

[a] Weak staining.

[b] Variable positivity in epithelial and myoepithelial cells of ducts and acinar epithelium (see text).

[c] Weak staining was observed in these tissues with immune, and with preimmune or keratin-absorbed serum and was therefore interpreted as nonspecific.

ficity of the staining was verified by parallel experiments using both neutralized antiserum and preimmune rabbit serum.

Keratin proteins were detected equally in all layers of the epidermis from stratum basale to stratum corneum, and in some skin adnexa including hair follicles, sweat gland ducts, and ducts of sebaceous glands (Fig. 2*A*). These observations are consistent with the findings of Sun and Green (28), except for the staining characteristics of the stratum granulosum. In the latter study, immunofluorescence with antikeratin antibodies demonstrated little fluorescence in the granular layer, presumably due to a quenching phenomenon. In addition, preimmune serum reacted weakly, but specifically, with this same layer. Immunoperoxidase staining exhibited neither of these characteristics. The stratum granulosum reacted as well with antikeratin antibodies as did the other layers, and none of the layers stained when using either preimmune or keratin-absorbed antiserum (Fig. 2*B*).

Other squamous epithelia such as oral mucosa, exocervix, and vaginal mucosa also stained strongly, although an unequal distribution of staining was found in the female genital tract. Whereas the basal epithelial cells of vaginal mucosa stained strongly, the upper layers (which contain variable amounts of glycogen in response to estrogen stimulation) reacted only weakly with the antiserum.

Epithelial-derived glands such as parotid gland (Fig. 2*D*), submaxillary gland, and pancreas (Fig. 2*H*), revealed relatively similar keratin distributions. The ductal epithelial cells of each of these glands, which are single layered, cuboidal, or columnar cells, contained significant amounts of intracellular keratin, whereas the acinar structures were uniformly negative. Staining within the parotid ductal system extended from the large striated ducts to the small intercalated ducts, despite the lack of detectable tonofilament bundles in these cells by electron microscopy (20, 23). Foci of squamous metaplasia in such ductal systems (*e.g.*, Fig. 2*H*), represented a focal proliferation of cells which stained intensely for keratin.

A different staining pattern was noted in the bronchial epithelium of the lung (Fig. 2*C*), cervix (Fig. 2*E*), and prostate (Fig. 2*F*). In general, these tissues contain an epithelium which is composed of superficial columnar cells and underlying basal or reserve cells. In the lung, the basal cells (and intermediate cells) contained significant amounts of keratin, unlike the ciliated and mucus columnar cells which lacked this protein. The cervix and prostate are bilayered, and only the basal cells located adjacent to the basement membrane contained keratin. Again, the superficial columnar cells were negative.

Squamous metaplasia is common in the bronchial and cervical epithelium and is believed to be related to the development of squamous cell carcinoma (2). In both of these tissues the appearance of squamous metaplasia appears to be due to the focal proliferation of the basal, keratin-positive epithelium. Areas of squamous metaplasia, regardless of tissue origin, stained strongly with antikeratin antibodies.

The staining characteristics of the breast were variable and dependent upon the specific fixative used. For example, formalin-fixed breast tissue displayed homogeneous staining of the entire ductal epithelium (consisting of the myoepithelium and the superficial ductal cells). In contrast, breast tissue fixed with B5 solution (4) shows an augmentation of myoepithelial cell staining relative to the overlying ductal cells (Fig. 2*G*). The latter findings are comparable to those described in previous immunofluorescence studies (8). All other tissues examined in this study had characteristic staining patterns which were independent of the fixation procedure.

As described for the rabbit (29), keratin was present in

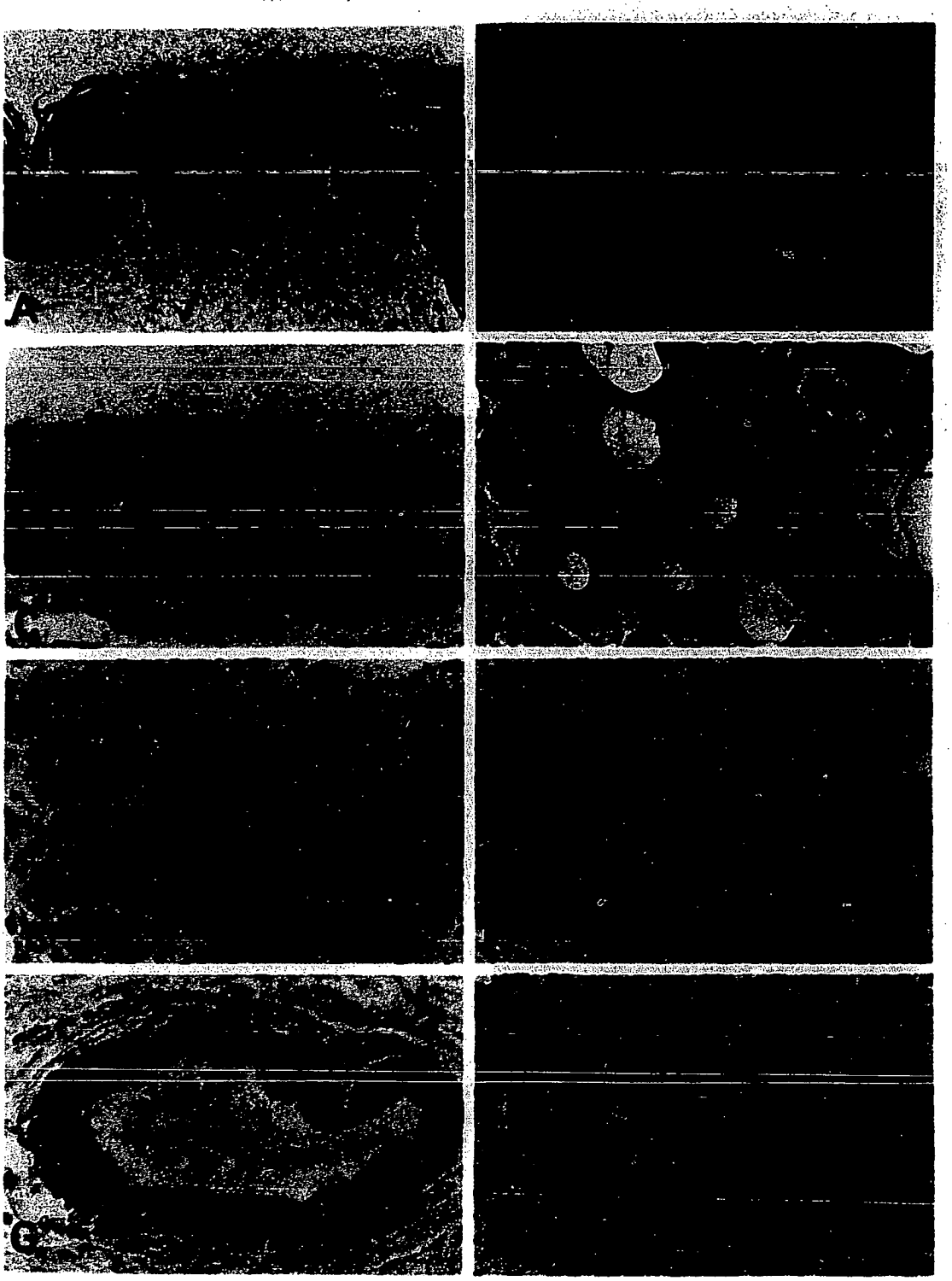

FIG. 2. Immunohistochemical localization of intracellular keratin in paraffin sections of normal human tissues. *A*, Skin. Keratin proteins (brown) present in all layers of epidermis. *B*, Skin. Absorption of immune serum with keratin protein completely abolishes staining reaction. *C*, Bronchus. Strong staining for keratin proteins in basal and intermediate cells. Ciliated and mucus columnar cells are devoid of detectable keratin proteins. *D*, Parotid. Keratin proteins identified in ductal epithelium. Acini are negative. *E*, Endocervix. Basal cells of endocervical glands demonstrate strong staining for keratin proteins. Mucus columnar cells are negative. *F*, Prostate. Staining for keratin proteins observed in basal cells of prostatic glands. Columnar cells are negative. *G*, Breast (B5 fixative). Prominent staining for keratin proteins in myoepithelial layer of duct, with weaker reaction in superficial epithelial cells. *H*, Pancreas. Staining for keratin proteins observed in ductal epithelium with increased staining intensity in areas of squamous metaplasia. Acini are negative. All tissues were counterstained with hematoxylin. Figure 2*A* to *C*, ×250; *D* and *H*, ×125; *E*, ×500; *F* and *G*, ×250.

the urothelium of the bladder and ureter and extended into the collecting duct system of the kidney. Keratin was absent in renal glomeruli. In the liver, only the bile ducts contained detectable keratin protein.

Staining characteristics of the gastrointestinal tract were equivocal. While there was a minimal degree of reactivity of such epithelia with the antikeratin antibodies, the staining was not abolished by the absorption of the antiserum with keratin proteins, and weak staining occurred even with the use of preimmune serum. We have interpreted these results as representing a nonspecific reaction.

DISCUSSION

The cytoplasm of mammalian cells contains a complex array of filamentous proteins which participate variously in the maintenance of cell shape and mobility (6), modulation of cell membrane protein movement (1, 19), mobility of chromosomes and cellular processes (10), cytokinesis (24), and possibly the regulation of cell proliferation (7). Keratin filaments, which are one type of cytoplasmic "intermediate" filament, are found principally in epidermal cells where they are believed to have a structural function. The recent immunologic detection of keratin proteins in many types of epithelia suggests that these filaments have a more diverse biologic distribution than previously recognized (8, 9, 27, 29). In addition, the strong reactivity of parotid ductal epithelium with antikeratin antiserum (Fig. 2*D*) and the absence of tonofilament bundles by electron microscopy (20) indicate that these epithelial cells contain abundant keratin proteins which are present in a nonaggregated form, ultrastructurally indistinguishable from other intermediate-sized filaments.

The present investigation represents the first comprehensive study to localize keratin proteins in human tissue and to identify specifically the basal cells of trachea, bronchi, prostate, and cervix as the predominant sites of keratin synthesis. Bronchial basal cells, which have been shown to contain numerous tonofilament bundles by electron microscopy (23), apparently represent the proliferating cells of the respiratory epithelium (3, 13). It is interesting to note that, during the differentiation of these basal cells into nondividing columnar cells, there is a concomitant loss of cellular keratin proteins by a mechanism not yet known. The histology of the normal human bronchus has recently been reviewed and examined by McDowell *et al.* (17). In this study, the cells commonly referred to by histologists as "intermediate" were shown to consist of a mixture of basal cells and small mucus granule cells which had similar appearances ultrastructurally, except that the small mucus granule cells often contained small, cytoplasmic periodic acid-Schiff-positive granules. Both cell types were considered to have a proliferative potential, based upon extrapolation from hamster experiments. Our studies demonstrate that both basal and intermediate cells (presumably including small mucus granule cells) contain prominent intracellular keratin protein. The recent finding that the analogous keratin-positive cells of the cervix also have a proliferative capacity (11) suggests that the basal cells of the prostate and breast may fulfill similar precursor-cell roles. The basal or reserve cells of the lung and cervix can proliferate in either a regulated fashion to produce regions of squamous cell metaplasia, or in an unregulated manner to produce squamous cell carcinoma (14).

Immunofluorescence studies with rabbit tissue have demonstrated the presence of keratin in both superficial and basal layers of trachea, bronchi, and cervical glands (29). Whether the difference between the rabbit and human tissues reflects species-related variation in gland architecture or keratin localization, or whether it results from differences in the sensitivity or resolution of the particular immunologic technique is unclear.

Keratin has a similar distribution pattern in the salivary glands, pancreas, and lung. These tissues arise developmentally by the ingrowth and sequential branching of an epithelial growth bud and, in each case, the keratin-containing cells are found only in the early branchings, that is, the ducts or bronchi. The terminal acini or alveoli lack keratin protein. Presumably, the process of gland or organ development results in the loss of keratin synthesis.

The detection of tonofilament bundles (aggregates of keratin filaments) by electron microscopy has been used for distinguishing poorly differentiated carcinomas from sarcomas and lymphomas. However, immunoperoxidase staining for keratin protein has significant advantages over electron microscopy for defining the epithelial nature of a tumor: a greater number of cells may be examined histologically; special fixation and processing are not necessary; the technique is rapid; and, most importantly, the keratin filaments need not necessarily be aggregated into characteristic bundles for their conclusive identification. Also, this immunohistochemical method affords excellent cellular detail and a permanent preparation. Attempts to extend the use of this technique to the differential diagnosis of benign and malignant human neoplasms as well as to the various "undifferentiated" tumors are in progress.

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